Volume 192, number 2 FEBS 3099 November 1985

Specific inhibition of mitochondrial protein synthesis influences the amount of complex I in mitochondria of rat liver and *Neurospora crassa* directly

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Received 27 August 1985

Specific inhibition of mitochondrial protein synthesis reduces the oxidation rate of NADH-linked substrates in rat liver as well as in *Neurospora crassa* mitochondria. The present study shows that this is due to the fact that inhibition of mitochondrial protein synthesis leads to a decrease of the concentration of active complex I. Therefore, these results demonstrate that at least one of the genes for the subunits of complex I is localized on mitochondrial DNA.

Protein synthesis inhibition NADH dehydrogenase Complex I Mitochondria (rat liver, Neurospora crassa*)*

1. INTRODUCTION

Mitochondria contain a small genome, which codes for several polypeptides, and a specific system to transcribe and translate this DNA [1]. Despite several differences in structure and organization between the mitochondrial (mt) genomes of lower eukaryotes and mammalian cells, the identity of the major polypeptide products is the same [2]. The mtDNAs, of e.g. rat liver and *Neurospora crassa (N. crassa),* both code for 3 subunits of cytochrome *c* oxidase, for apocytochrome *b* and for 1 subunit of the ATP synthase complex. These polypeptides are all part of proteins located in the inner mt membrane and are involved in oxidative phosphorylation.

MtDNA contains also several unassigned reading frames (URFs). Sequence analysis of these URFs in various mtDNAs [3] showed that their possible translation products, which are all hydrophobic, are conserved among species. It may be, therefore, that some URF products are also membrane-bound and are part of the oxidative phosphorylation system as well.

Recently, a number of authors suggested that some of the URFs code for subunits of complex I of the respiratory chain. Evidence for this assumption was found in mammalian [4,5] as well as in lower eukaryotic cells [6].

We have shown that long-term inhibition of mt protein synthesis reduces the oxidation rate of various NADH-linked substrates in mammalian mitochondria and cells [7]. This clearly supports a possible role of the mt genome in complex I formation, but alternative explanations are also possible. In this study it will be shown that prolonged inhibition of mt protein synthesis leads to decreased amounts of complex I directly. This shows, therefore, that the formation of complex I depends on the expression of mtDNA.

2. MATERIALS AND METHODS

2.1. *Inhibition of mt protein synthesis*

Oxytetracycline (OTC) was given by continuous i.v. infusion to Wistar rats [8] in amounts sufficient to maintain serum levels at 4–6 μg/ml. For studies on lower eukaryotes, *N. crassa* 89601, an

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00145793/85/$3.30

inositol-requiring strain, was used; 1 l cultures were grown at 30°C on a rotary shaker. Chloramphenicol (CAP, final concentration 3.2 mg/ml) or OTC (final concentration 0.6 mg/ml) were added to the cultures during the early exponential phase of growth. Under the conditions used, mt protein synthesis is inhibited selectively in both rat liver and *N. crassa*.

2.2. *Preparation of mitochondria*

Mitochondria were isolated from homogenates by differential centrifugation according to standard procedures [9,10]. The medium was 0.25 M sucrose for rat liver and 0.44 M sucrose, 10 mM Tris, 0.5 mM EDTA and 2 mM $MgCl_2$, pH 7.4, for *N. crassa* mitochondria. *N. crassa* hyphae were homogenized by grinding with sand in a mortar.

2.3. *Analytical methods*

The oxygen consumption of either freshly prepared (*N. crassa*) or freeze-thawed (rat liver) mitochondria was measured at 30°C with a Clark oxygen electrode. The medium contained 0.25 M sucrose, 10 mM Tris-HCl, 2.5 mM potassium phosphate, 50 mM KCl, 2.5 mM $MgCl_2$ and 1.0 mM EDTA, pH 7.4.

Glutamate plus malate (final concentration, respectively, 10 and 2 mM) were added to study the NADH-linked oxidation in rat liver mitochondria, and pyruvate plus malate (respective final concentration, 10 and 2 mM) were used to this end in studies in *N. crassa*. Addition of the substrates was sufficient to induce respiration, since under our experimental conditions only uncoupled respiration was measurable. After a few minutes piericidin A, dissolved in 90% dimethyl sulfoxide (DMSO) was added. As DMSO itself appeared to inhibit the oxidation rate, separate runs, during which piericidin was added in a fixed volume of DMSO, were performed for every piericidin concentration to be tested.

The amount of cytochrome pigments in the various mitochondria was calculated from difference spectra with the aid of the extinction coefficients and wavelength pairs as described by Estabrook and Holowinsky [11]. For cytochrome *c* of *N. crassa* mitochondria, however, an extinction coefficient of 18.7 $mM^{-1} \cdot cm^{-1}$ [12] was used. Protein was determined with a modified Lowry method [13].

3. RESULTS AND DISCUSSION

Piericidin A inhibits complex I (NADH:ubiquinone oxidoreductase or NADH dehydrogenase) activity in a specific and equimolar way [14]. For this reason, it should be possible to construct dose vs response curves of piericidin and NADH-linked respiration and subsequently to calculate the complex I content of isolated mitochondria. Fig.1 shows such titration curves for mitochondria isolated from the livers of rats treated during various periods with OTC. These data allow several conclusions. It is known that the respiratory rate with glutamate plus malate (and that of various other NADH-linked substrates) is lower than that of, e.g. succinate. Since this holds also for the uncoupled or Ca^{2+}-induced respiration, it is not the ATP synthetase which is rate-limiting for NADH-linked oxidation. Fig.1 makes clear, however, that complex I is also not rate-limiting for

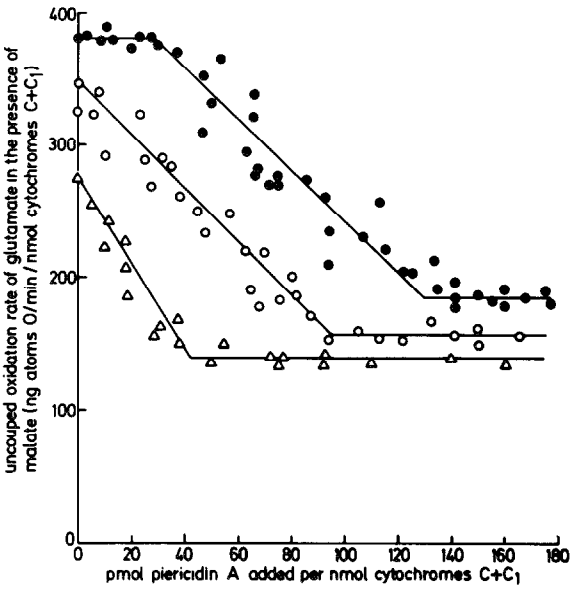

Fig.1. Piericidin A titration curves of glutamate-induced respiration in rat liver mitochondria. The respiratory rate was measured in the presence of varying amounts of piericidin, added in 10 μl of 90% DMSO. Every point represents one measurement; for every curve the mitochondria of 3 different rats were used. The slope of the curves was calculated by linear regression analysis, the correlation coefficient was minimally 0.92. (●) Control mitochondria; (○) OTC-treated, cytochrome aa_3 content 56%; (△) OTC-treated, cytochrome aa_3 content 30%.

glutamate oxidation, because the piericidin A titration curve shows a distinct lag phase in studies with control rat liver mitochondria. Therefore, either the maximal rate of the transport of electrons derived from NADH is determined by some component, located between complex I and complex III of the respiratory chain or the glutamate dehydrogenase activity is limiting for glutamate oxidation.

The situation changes, however, if mitochondria from liver of long-term OTC-treated rats are studied. As has been described [7], the oxidation rate of NADH-linked substrates declines as mt protein synthesis has been inhibited for a longer period. This observation cannot be explained on the basis of reduction of the amounts of respiratory chain components already known to be of (partly) mitochondrial biogenetic origin. The data shown in fig.1 offer, however, a clue. It can be seen that the lag-phase observed in the dose vs response curve of the control mitochondria has disappeared in the liver mitochondria of OTC-treated rats. We interpret this to mean that complex I activity becomes rate-limiting for NADH-linked oxidation if the amount of mtDNA gene products has been reduced to a certain level by inhibition of mt protein synthesis.

Furthermore, it is possible to calculate the amount of functional complex I from the amount of piericidin A needed to inhibit glutamate induced respiration maximally. The addition of an excess of piericidin never led to complete inhibition of respiration, at 10 nmol piericidin added per nmol cytochromes $c + c_1$ the oxidation rate still remained at about 40% of its original value. However, we took no precautions to prevent the indirect oxidation of glutamate (and malate) via the formation of succinate. The minimal amount of piericidin A which gave no significant further reduction of the oxidation rate was therefore used to calculate the complex I content in the various mitochondria. These data, as well as the amounts of cytochromes $c + c_1$ and of cytochrome aa_3 are given in table 1. It is obvious that the amount of complex I decreased in a way comparable to that of cytochrome aa_3 by long-term inhibition of mt protein synthesis. The same conclusion can be drawn from the results of EPR spectroscopy studies on liver submitochondrial particles of OTC-treated rats. These experiments (performed in collaboration with Dr S. Albracht, and to be published elsewhere) showed that also the amount of the iron-sulfur clusters characteristic of complex I, decline in a way comparable to that of

Table 1

Effect of inhibition of mt protein synthesis on the amounts of cytochromes $c + c_1$, of cytochrome aa_3 and of complex I in mitochondria from rat liver and *N. crassa*

	Cytochrome aa_3 / cytochromes $c + c_1$	Complex I / cytochromes $c + c_1$	Cytochrome $c + c_1$ (pmol/mg protein)	Cytochrome aa_3 (pmol/mg protein)	Complex I (pmol/mg protein)
Rat liver					
control	0.363 (100%)	0.129 (100%)	393 (100%)	143 (100%)	51 (100%)
OTC	0.203 (56%)	0.095 (73%)	391 (100%)	79 (55%)	37 (73%)
OTC	0.109 (30%	0.042 (32%)	362 (92%)	40 (28%)	15 (30%
N. crassa					
control*	0.236 (100%)	0.070 (100%)	1173 (100%)	277 (100%)	82 (100%)
OTC/CAP	0.174 (73%)	0.055 (79%)	1165 (99%)	203 (72%)	64 (78%)
OTC/CAP	0.122 (50%)	0.031 (44%)	1388 (118%)	169 (55%)	43 (52%)
CAP	0.0 (0%)	0.0 (0%)	1561 (133%)	0 (0%)	0 (0%)

* Mid-exponential phase of growth

The data refer to the mitochondria used in figs 1 and 2 and show the relative and absolute amounts of cytochromes $c + c_1$, of cytochrome aa_3 and of complex I in the mitochondria which were used for the piericidin titration studies. The cytochrome content was calculated from spectral analysis; the complex I content was deduced from the data shown in figs 1 and 2

cytochrome aa_3. Since the direct link between mt protein synthesis and the formation of cytochrome aa_3 is well known, the existence of a similar link between mt protein synthesis and the formation of complex I, is therefore strongly supported.

In view of the resemblance in mt genes, the same direct relationship between mt protein synthesis and the formation of complex I can be expected for other organisms as well. For this reason, we performed also a number of studies with mitochondria isolated from *N. crassa* cultures which were grown for various periods in the presence of OTC or CAP. First, we investigated whether the growth stage influenced the complex I content of *N. crassa* mitochondria, because it has been described [15] that the activity of complex I increased markedly during the late exponential and stationary phases of growth. Our results (not shown) confirm this observation. Titration curves showed that mitochondria from *N. crassa* harvested in the stationary growth phase possess nearly twice the amount of complex I as the mitochondria isolated from cultures in the early or mid-exponential phase. Between the latter 2 growth stages the variations in complex I content were, however, small. To exclude the influence of the growth or differentiation phase on the measurement of mt complex I concentrations, control mitochondria were isolated from cultures in the mid-exponential growth phase and CAP or OTC were generally added in a way that mt protein synthesis was inhibited for various periods during the early exponential phases of growth, whereas mitochondria from those cultures again were prepared in the mid-exponential stage. The results of the several piericidin titration studies performed with mitochondria from cultures grown in the presence of OTC or CAP were combined on the basis of their cytochrome aa_3 content.

Fig.2 shows the titration curves obtained with control and with mitochondria in which protein synthesis had been inhibited. The curve calculated for the mitochondria prepared from control cultures reveals again a lag phase, which implies that complex I is not rate-limiting for the oxidation of pyruvate in *N. crassa*. In mitochondria from cells cultured in the presence of inhibitors of mt protein synthesis, this lag phase is absent, however, and the oxidation rate of pyruvate decreases as the concentration of the products of mt protein synthesis becomes more reduced. Furthermore, the percentage of the pyruvate induced respiration which is sensitive to piericidin is also a function of the amount of mt gene products present. In control mitochondria, the oxidation of pyruvate can be reduced by piericidin to about 20%, implying that 80% of the observed respiration depends on the activity of complex I. At cytochrome aa_3 levels of 73, 50 and about 0% of the control value, the piericidin-sensitive oxidation shifts to 56, 45 and 0%, respectively. This change towards a type of respiration which is independent of complex I, is based on the activity of a alternate oxidase system which is known to be induced in *N. crassa* and other fungi [16] during inhibition of mt protein synthesis. This

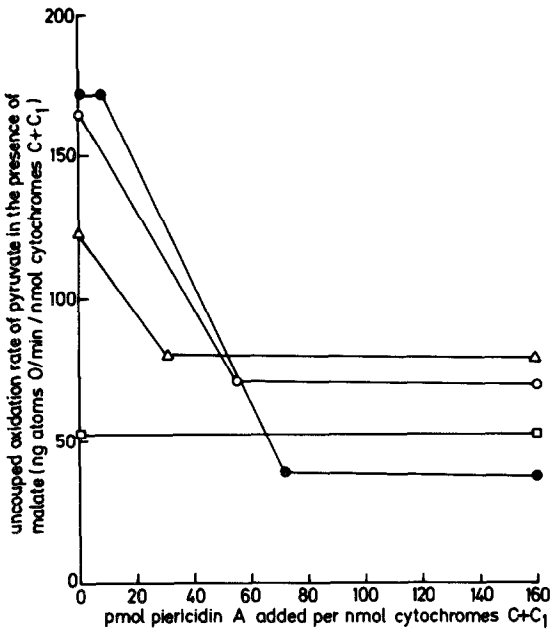

Fig.2. Piericidin A titration curves of pyruvate-induced respiration in *N. crassa* 89601 mitochondria. The respiratory rate was measured in the presence of varying amounts of piericidin, added in 3 μl of 90% DMSO. The individual points have been left out for reasons of clarity, per curve, however, minimally 20 measurements were done, with the mitochondria of at least 2 different cultures. The slope of the curves was calculated by linear regression analysis, the correlation coefficient was never below 0.93. (●) Control mitochondria, mid-exponential phase of growth; (○) OTC- or CAP-treated, cytochrome aa_3 content 73%; (△) CAP-treated, cytochrome aa_3 content 50%; (□) CAP-treated, cytochrome aa_3 content not detectable.

oxidase system is less efficient in ATP synthesis, which also explains why the *N. crassa* cultures in which the cytochrome aa_3 content was reduced showed a retarded growth. Finally, it can be seen in table 1 that the amount of functional complex I, calculated from the piericidin titration curves, declines in a way comparable to that of cytochrome aa_3 during inhibition of mt protein synthesis. The effect of inhibition of mt protein synthesis on the complex I content of mitochondria is, in this respect, identical in rat liver and in *N. crassa*.

In summary here we confer direct evidence that the formation of complex I of the respiratory chain is dependent on mt protein synthesis. Therefore, our results add essential proof that the genes for one or more subunits of complex I are localized on mtDNA.

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the Dutch Foundation for Cancer Research, K.W.F. Piericidin A was a generous gift from Drs S. de Vries and S. Albracht, University of Amsterdam, Amsterdam, The Netherlands. Mrs J.C. Dallinga-de Jonge and Mrs T. Melis are thanked for their analytical assistance and Mrs R. Kuperus for typing the manuscript.

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